PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/86, A61K 35/76, C12N 7/00, 15/34, C07K 14/075

A1

(11) International Publication Number: **WO 00/56909**

(43) International Publication Date: 28 September 2000 (28.09.00)

(21) International Application Number: PCT/GB00/01142

(22) International Filing Date: 24 March 2000 (24.03.00)

(30) Priority Data:
9906815.7 24 March 1999 (24.03.99) GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTI–NEOPLASTIC VIRAL AGENTS COMPRISING TOXIN GENE UNDER CONTROL OF TUMOUR CELL–DERIVED TRANSCRIPTION FACTORS

(57) Abstract

A viral DNA construct, and virus encoded thereby, is provided having one or more tumour specific transcription factor binding sites in place of one or more wild type transcription factor binding sites operatively positioned in the promoter region which controls expression of early genes responsible for viral nucleic acid replication. Preferred constructs place the tumour specific transcription factor binding sites in operative relation to DNA polymerase, DNA terminal protein and/or DNA binding protein. Compositions and constructs contained therein are provided, particularly for use in therapy. Methods of treating patients for neoplasms are also provided.

ANTI-NEOPLASTIC VIRAL AGENTS COMPRISING TOXIN GENE UNDER CONTROL OF TUMOUR CELL-DERIVED TRANSCRIPTION FACTORS

The present invention provides viral agents that have application in the treatment of neoplasms such as tumours, particularly tumours derived from colon cells, more particularly liver tumours that are metastases of colon cell primary tumours. Still more particularly are provided replication efficient adenovirus constructs that selectively replicate in response to transcription activators present in tumour cells, these factors being present either exclusively or at elevated levels in tumour cells as compared to other cells, and thus which lead to tumour cell death and cell lysis.

By injecting these viral agents locally into the liver it is possible to treat liver metastases, which are a major cause of morbidity in colon cancer patients. Applications beyond this, e.g. to other sites and other tumours, such as colorectal cancers and melanomas, are also provided.

Colon cancer presents with locally advanced or metastatic disease in the majority of patients. Most patients are left with liver metastases as the only site of disease after resection of the primary tumour. Partial liver resection only cures about 10% of patients, while in patients with multiple metastases in both liver lobes resection is not feasible and loco-regional or systemic treatment with chemotherapy is indicated (Labianca et al., 1997). Systemic chemotherapy with 5-fluorouracil and leucovorin or irinotecan will produce response rates of only 20% (Cunningham et al., 1998; Stupp et al., 1998).

Locoregional chemotherapy of the liver has been explored for over 15 years. Most liver metastases are supplied with blood by the hepatic artery, so intra-arterial hepatic chemotherapy (IAHC) allows for much higher exposure of the metastases to cytotoxic drugs. The high extraction rate of normal liver decreases the systemic drug concentration resulting in less toxicity with IAHC having been shown repeatedly to give response rates over 60% (Kemeny et al., 1987; Kemeny et al., 1992; Patt and Mavligit, 1991).

Specific defects in tumour cells make it possible to devise rational strategies for targeting tumour cells without harming normal cells. With the exception of anti-

A more promising approach expressing wild type p53 using an adenovirus, Ad-CMV-p53, has been demonstrated clinically in head and neck cancer and is currently under investigation in lung, colon and liver cancer (Clayman et al., 1998). Adenoviruses are relatively stable, can be produced at high titres, and can infect both quiescent and dividing cells of many different types. Overall, Ad-CMV-p53 appears exceptionally non-toxic but probably ineffective as a single agent; hence, there is a place for more aggressive second generation viruses.

Further target specific defects are mutations of p16, cdk4, cyclin D or Rb (Bartek et al., 1997) in the retinoblastoma pathway which cause loss of G1/S control and essentially all tumours have these. The only significant exception is colon cancer, where mutations in the Rb pathway itself are rare. The net result of these defects is increased E2F activity, which means that tumours can be selectively targeted by viruses expressing toxic genes from E2F-regulated promoters. This has been demonstrated using an adenovirus expressing the HSV thymidine kinase gene from such a promoter (Parr et al., 1997); cells containing Rb-pathway mutations express tk and can be killed by ganciclovir. Such an approach relies on an increase in the activity of specific transcription factors in tumour cells.

The rational basis for tumour targeting is better understood for non-replicating E2F-targeting viruses than it is for p53, but both are still single hit approaches and it is very difficult to see how they can ever be used for more than treatment of local disease. The tumour burden in late stage disease is around 10^{12} cells, so while at an effective multiplicity of infection of one treatment would be feasible, in practice biodistribution and receptor problems mean that many orders of magnitude higher multiplicities are required.

One elegant way to circumvent this limitation is to recruit the immune system to kill the tumour cells. The role of the gene therapy virus is simply to provoke or reinforce the immune response. There is abundant evidence that tumours express new antigens, but in cancer patients the immune system has clearly failed to prevent tumour formation. Many currently attempted techniques target single antigens, eg production of cytotoxic T cells against MAGE antigens in melanoma, but the goal for

amount of dl 1520 virus injected is comparable for therapy with Ad-CMV-p53. This means that the virus is not performing as expected for a replicating virus with the reasons for this again probably quite complex. Adenoviruses normally produce superficial mucosal infections which are spread by droplets containing infected cells. Infected cells retain progeny virus. Lack of effective virus release from lysed cells will militate against the production of deep, spreading infection, which is the goal if virus is to penetrate to all parts of the tumour.

Rational targeting of E2F defects is complicated by the fact that as part of its life cycle the adenovirus already produces proteins (E1A and E4 orf 6/7) which target E2F. Since E1A and orf 6/7 are multifunctional proteins the effect of E1A and orf 6/7 mutations is complex and unpredictable.

In addition to E2F and p53, there are four transcription factors whose activity is known to increase in tumours. They are Tcf4, RBPJκ and Gli-1, representing the endpoints of the wnt, notch and hedgehog signal transduction pathways (Dahmane et al., 1997; Jarriault et al., 1995; van de Wetering et al., 1997) and HIF1alpha, which is stabilised by mutations in the Von Hippel Lindau tumour suppressor gene (Maxwell et al 1999). Mutations in APC or β-catenin are universal defects in colon cancer (Korinek et al., 1997; Morin et al., 1997); but they also occur at lower frequency in other tumours, such as melanoma (Rubinfeld et al., 1997). Such mutations lead to increased Tcf activity in affected cells. The hedgehog pathway is activated by mutations in the patched and smoothened proteins in basal cell cancer (Stone et al., 1996; Xie et al., 1998). Notch mutations occur in some leukaemias (Ellisen et al., 1991). Telomerase activation is one of the hallmarks of cancer (Hanahan D. and Weinberg RA. The hallmarks of cancer. Cell. 100, 57-70, 2000) and results from increased activity of the telomerase promoter, although the mechanism is unknown. According to Cong YS et al (1999, HMG 8, 137-42) the elements responsible for promoter activity are contained within a region extending from 330 bp upstream of the ATG to the second exon of the gene and thus this sequence is a further suitable promoter sequence for use in the viral constructs and viruses of the invention.

Most importantly and advantageously, the present inventors have made possible to target tumour cells with a virus encoding only wild type viral proteins, whose expression is specifically regulated by transcription factors preferentially or exclusively activated in tumour cells. Preferred virus encodes a full set of wild type proteins. Such virus can be used to better effect than prior art viral agents which have relied on mutation of viral proteins or targeting of cells of a particular tissue origin, eg. prostate.

Thus in a first aspect of the present invention there is provided a viral DNA construct encoding for a virus that is capable of replication in a human or animal tumour cell type and causing tumour cells of that type to die characterised in that the construct comprises one or more selected transcription factor binding sites together with, and operatively positioned such as to promote expression of, open reading frames encoding early viral proteins, the protein products of those reading frames being mechanistically directly involved in viral construct nucleic acid replication wherein the selected transcription factor binding sites are for a transcription factor the level or activity of which is increased in a human or animal tumour cell relative to that of a normal human or animal cell of the same type.

Preferred constructs of the invention have a nucleic acid sequence corresponding to that of a wild type virus sequence characterised in that it has one or more wild type transcription factor binding sites replaced by one or more selected transcription factor binding sites, these sites being operatively positioned in the promoter region which controls expression of early genes such as to promote expression of the open reading frame of the gene, the protein products of those genes being mechanistically directly involved in viral nucleic acid replication

wherein the selected transcription factor binding sites are for a transcription factor the level or activity of which is increased in a human or animal tumour cell relative to that of a normal human or animal cell of the same type.

Preferably the viral DNA construct is characterised in that the sites which replace the wild type transcription factor sites controlling expression of said early

happen at some reasonable frequency; about 1 in 100 million cells, a mutation rate of 1 in 10^8. If you have to delete two receptors you multiply the probabilities; ie. loss of both will occur in 1 in 10^{16} cells. A tumour contains between 10^9 and 10^{12} cells. Hence resistance is less likely to develop if a virus uses more than one receptor. One fibre protein in Ad40 and 41 uses CAR whilst the receptor used by the other is as yet unknown.

Advantageously the use of the constructs of the invention, particularly in the form of viruses encoded thereby, to treat liver metastasis is relatively non-toxic compared to chemotherapy, providing good spread of virus within the liver aided by effective replication.

Preferred viral constructs of the invention are derived from adenovirus or parvovirus genomic DNA, more preferably adenovirus genomic DNA, and are mutated such that transcription of essential viral genes encoded by the E2 viral transcription unit is made dependent on the presence of oncogenic mutations in tumour cells. Preferably only cells containing these oncogenic mutations can activate transcription of the viral E2 genes. Since the E2 unit encodes the viral DNA polymerase, DNA terminal protein and DNA binding protein, the virus can only replicate in tumour cells. It is preferred that the E2 early promoter transcription factor binding site is replaced by the tumour cell specific transcription factor binding site.

Preferred tumour specific transcription factor binding sites that are used in place of wild type sites are those described above as Tcf-4, HIF1alpha, RBPJκ and Gli-1 sites, and a fragment of the telomerase promoter conferring tumour-specific transcription. A most preferred transcription factor binding site is that which binds Tcf-4, such as described by Vogelstein et al in US 5,851,775 and is responsive to the heterodimeric β-catenin/Tcf-4 transcription factor. As such the transciption factor binding site increases transcription of genes in response to increased β-catenin levels caused by APC or β-catenin mutations. The telomerase promoter is described by Wu KJ. et al (1999, Nat Genet 21, 220-4) and Cong YS. et al (1999 HumMol Genet 8, 137-42). A further preferred binding site is that of HIF1alpha, as described by Maxwell PH. et al, (1999 Nature 399, 271-5). One may use a HIF1alpha-regulated

In a preferred embodiment of the first aspect of the present invention the inventors have replaced a short region in the E2 early promoter, which is not overlapped by coding sequence, with multiple Tcf binding sites, more preferably 3 or 4 such sites, and most preferably 4 sites. One resulting preferred viral construct and encoded virus are referred to herein as Ad-Tcf3, having 3 such sites, and the virus expresses E2 gene products and replicates better in colon than in lung tumour cells (see Examples). This shows that mutations of the type described can modify the activity of the E2 promoter in the desired way without untoward effects on other aspects of the viral life cycle. This is not obvious *a priori* because, as well as encoding the E2 promoter, this region is transcribed and retained in the 5'-untranslated region of the pVIII protein RNA, it is transcribed but spliced out of the L5 late RNA and it forms part of the 3'-untranslated region of the 33k protein RNA.

Although Ad-Tcf3 replicates better in colon than lung tumour cells, the difference is only around ten-fold. LGC, a virus of the invention combining the E2 mutations in Ad-Tcf3 with an E1B 55K deletion, shows around 1000-fold selectivity for colon cancer cells. This demonstrates that E2 promoter activity can be made limiting for viral replication, because the identical virus with the normal E2 promoter (LGM) shows no specificity for colon cells. For a colon-targeting strategy this is an important result because it means a colon-specific virus can be made by titrating the E2 promoter activity.

The probable explanation for the selectivity of LGC is that the E1B 55k protein is required for nuclear export of late viral RNAs, including the E2 DBP late RNA, and that the E2 RNA export defect in E1B 55K-deficient viruses can be overcome by increasing E2 RNA production by inserting Tcf sites in place of the normal transcription factor binding sites in the E2 promoter.

One method for increasing the specificity of the AdTcf3, and similar 3xTcf driven E2 viruses of the invention for colon tumours is to reduce its E2 promoter activity in non-colon cells. One possible way to do this is to alter the number of Tcf sites. Reduction in the number of Tcf sites to two could reduce non-specific leakiness, but since Tcf promoters are actively repressed in non-colon cells by groucho (Fisher

below). This is designed to abolish binding of CCAAT box binding factors without changing the 100k protein sequence. Additional silent mutations in the other footprints can be used to reduce activity further

An alternative or additional mutation possible is to regulate expression of E1B transcription by mutating the E1B promoter. This has been shown to reduce virus replication using a virus in which a prostate-specific promoter was used to regulate E1B transcription (Yu, D. C., et al 1999 Cancer Research 59, 1498-504). A further advantage of regulating E1B 55K expression in a tumour-specific manner would be that the risk of inflammatory damage to normal tissue would be reduced (Ginsberg, H. S., et al 199 PNAS 96, 10409-11). The inventors have produced viruses with Tcf sites replacing the E1B promoter Sp1 site to test this proposition.

Further embodiments include the following possible modifications. To further restrict replication one can insert further tumour specific, eg. Tcf, sites in the E1A promoter. To achieve regulation by inserting short oligonucleotides, one must delete the existing regulatory sequences in the E1A promoter. This requires simultaneous mutation of both inverted terminal repeats and transfer of the packaging signal elsewhere, eg. to the E4 region. In contrast with, for example, the Calydon viruses, the design of the present inventors viruses means that, despite retaining a full complement of adenoviral genes, spare packaging capacity is available, which can be used to express conditional toxins, such as the prodrug-activating enzyme HSV thymidine kinase (tk). This could be expressed for example from the E3 promoter, whose activity is regulated in some of the viruses, to provide an additional level of tumour targeting. Alternatively, it could be expressed from a constitutive promoter to act as a safety feature, since ganciclovir would then be able to kill the virus. Constitutive tk expression in an E1B-deficient virus also increases the tumour killing effect, albeit at the expense of replication (Wildner, O., et al 1999 Gene Therapy 6, 57-62). An alternative prodrug-activating enzyme to express would be cytosine deaminase (Crystal, R. G., et al 1997 Hum Gene Ther 8, 985-1001), which converts 5FC to 5FU. This has advantage because 5FU is one of the few drugs active on liver metastases, the intended therapeutic target, but produces biliary sclerosis in some patients.

;17-20, 9474; Davison et al J. Mol. Biol (1993) 234(4) 1308-16; Kidd et al Virology (1990) 179(1) p139-150; all of which are incorporated herein by reference.

In a second aspect of the invention there is provided the viral DNA construct of the invention, particularly in the form of a virus encoded thereby, for use in therapy, particularly in therapy of patients having neoplasms, eg. malignant tumours, particularly colorectal tumours and most particularly colorectal metastases. Most preferably the therapy is for liver tumours that are metastases of colorectal tumours.

In a third aspect there is provided the use of a viral DNA construct of the invention, particularly in the form of a virus encoded thereby, in the manufacture of a medicament for the treatment of neoplasms, eg. malignant tumours, particularly colorectal tumours and most particularly colorectal metastases. Most preferably the treatment is for liver tumours that are metastases of colorectal tumours.

In a fourth aspect of the invention there are provided compositions comprising the viral DNA construct of the invention, particularly in the form of a virus encoded thereby, together with a physiologically acceptable carrier. Such carrier is typically sterile and pyrogen free and thus the composition is sterile and pyrogen free with the exception of the presence of the viral construct component or its encoded virus. Typically the carrier will be a physiologically acceptable saline.

In a fifth aspect of the invention there is provided a method of manufacture of the viral DNA construct of the invention, particularly in the form of a virus encoded thereby comprising transforming a viral genomic DNA, particularly of an adenovirus, having wild type transcription factor binding sites, particularly as defined for the first aspect, controlling transcription of genes the protein products of which are directly mechanistically involved in viral nucleic acid replication, such as to operationally replace these sites by tumour specific transcription factor binding sites, particularly replacing them by Tcf transcription factor binding sites. Operational replacement may involve partial or complete deletion of the wild type site. Preferably the transformation inserts two or more, more preferably 3 or 4, Tcf-4 transcription factor binding sites. More preferably the transformation introduces additional mutations to one or more residues in the NF1, NFκB, AP1 and/or ATF binding sites in the E3

Although it can be used to make E1-replacement viruses, and the inventors have constructed YAC/BACs allowing cycloheximide selection of desired recombinants in the yeast excision step to simplify this task, the main strength of the approach is that it allows introduction of mutations at will throughout the viral genome. Further details of the YAC/BAC are provided by the inventors as their contribution to Gagnebin et al (1999) Gene Therapy 6, 1742-1750) which is incorporated herein by reference. :Sequential modification at multiple different sites is also possible without having to handle large DNA intermediates in vitro.

The adenovirus strain to be mutated using the method of the invention is preferably a wild type adenovirus. Conveniently adenovirus 5 (Ad 5) is used, as is available from ATCC as VR5. The viral genome is preferably completely wild type outside the regions modified by the method, but may be used to deliver tumour specific toxic heterologous genes, eg. p53 or genes encoding prodrug-activating enzymes such as thymidine kinase which allows cell destruction by ganciclovir. However, the method is also conveniently applied using viral genomic DNA from adenovirus types with improved tissue tropisms (eg. Ad40 and Ad41).

In a sixth aspect of the present invention there is provided a method for treating a patient suffering from neoplasms wherein a viral DNA construct of the invention, particularly in the form of a virus encoded thereby, is caused to infect tissues of the patient, including or restricted to those of the neoplasm, and allowed to replicate such that neoplasm cells are caused to be killed.

The present invention further attempts to improve current intra-arterial hepatic chemotherapy by prior administration of a colon-targeting replicating adenovirus. DNA damaging and antimetabolic chemotherapy is known to sensitise tumour cells to another replicating adenovirus in animal models (Heise et al., 1997). For example, during the first cycle the present recombinant adenovirus can be administered alone, in order to determine toxicity and safety. For the second and subsequent cycles recombinant adenovirus can be administered with concomitant chemotherapy. Safety and efficacy is preferably evaluated and then compared to the first cycle response, the patient acting as his or her own control.

tumour activity over at least some of those lacking this adverse effect. In this event it is appropriate that an immuno-suppressive, anti-inflammatory or otherwise anti-cytokine medication is administered in conjunction with the virus, eg, pre-, post- or during viral adminstration. Typical of such medicaments are steroids, eg, prednisolone or dexamethasone, or anti-TNF agents such as anti-TNF antibodies or soluble TNF receptor, with suitable dosage regimes being similar to those used in autoimmune therapies. For example, see doses of steroid given for treating rheumatoid arthritis (see WO93/07899) or multiple sclerosis (WO93/10817), both of which in so far as they have US equivalent applications are incorporated herein by reference.

The present invention will now be described by way of illustration only by reference to the following non-limiting Sequences, Figures and Examples. Further embodiments falling within the scope of the claims will occur to those skilled in the art in the light of these.

SEQUENCE LISTING

SEQ ID No 1 is the DNA sequence of Ad 5 with the E2 and E3 transcription site mutated in accordance with the invention as shown in Figure 2 with 4xTcf inserted in place of wild type E2 promoter.

SEQ ID No 2 is the partial amino acid sequence of the 33k protein encoded in SEQ ID No 1 unaffected by the insertion of Tcf instead of the wild type E2 promoter.

SEQ ID No 3 is the partial amino acid sequence of the pVIII protein encoded in SEQ ID No 1 unaffected by the insertion of Tcf instead of the wild type promoter.

SEQ ID No 4 is of wild type Adenovirus VR5 in the E2/E3 region.

SEQ ID No 5 is the DNA sequence of E2 late promoter as changed in a preferred virus of the invention as shown in Figure 4.

SEQ ID No 6 is a partial amino acid sequence 100k protein encoded in SEQ ID No 5 that is unaffected by the mutations in the late promoter.

SEQ ID No 7 is the DNA sequence of E1B promoter as mutated in a preferred virus of the invention as shown in Figure 20.

FIGURES

Figure 1 shows wild type E2 region position where transcription factor sites are inserted in preferred adenoviruses of the invention..

Figure 2 shows E3 region changes to wild type virus in a preferred virus

Figure 3 shows E2/E3 region of wild type virus.

Figure 4 shows E2 late promoter changes to wild type in a further preferred virus.

Figure 5 shows a diagrammatic representation illustrating steps taken in production of viruses of the invention.

Figure 6 is a diagrammatic representation of plasmid pNKBAC39.

Figure 7 is a diagrammatic representation of plasmid p680.

Figure 8 is a slot blot of virus infected cells probed with adenovirus DNA (A) or human genomic DNA (loading control B).

Figure 9 is a graphic representation of results of quantatative PCR of viral DNA from virus infected cells.

Figure 10 is a histogram showing E2 early expression in SW 480 and H1299 cell lines infected with wild type Ad5, and vMB12, vMB13 and vMB14 of the invention as measured by Taqman RT-PCR.

Figure 11 is a histogram showing E3 expression in SW480 and H1299 cell lines infected with wild type Ad5, and vMB12, vMB13 and vMB14 of the invention as measured by Taqman RT-PCR.

Figure 12 is a histogram showing DNA replication of wild type Ad5, and vMB12, vMB13 and vMB14 of the invention in SW480 and H1299 cell lines as measured by Taqman RT-PCR.

Figure 13 is a histogram showing burst size (arbitrary units) with wild type Ad5, and vMB12, vMB13 and vMB14 of the invention in SW480, H1299 and WI38 (fibroblast) cell lines.

Figure 14 is a graph showing CPE results % v particles/cell of wild type Ad5, and vMB12, vMB13 and vMB14 of the invention in SW480

Figure 15 is a graph showing CPE results % v particles/cell of wild type Ad5, and vMB12, vMB13 and vMB14 of the invention in H1299.

YAC/BAC (pMB19): The adenovirus genome was modified in a large plasmid with a bacterial artificial chromosome (F') replication origin, a yeast centromere and replication origin, and selectable markers for yeast and bacteria (HIS3, chloramphenicol resistance gene).

Ad5 YAC/BAC (pMB20): Genomic DNA was prepared from adenovirus type 5 obtained from ATCC (VR5). Small terminal fragments were amplified by PCR and cloned into the YAC/BAC. The vector was linearised at a site between the two terminal Ad5 fragments and transfected into yeast together with full length Ad5 genomic DNA. The plasmid was recircularised by homologous recombination (gap repair), giving full length Ad5 genomic DNA cloned in the YAC/BAC.

E1B::GFP fusion (pMB25): An Ad5 fragment containing the part of E1B 55k which overlaps E1B 19k was cloned by PCR and fused to the 5'-end of EGFP. This was then embedded in a larger Ad5 fragment, so that the E1B::GFP fusion was flanked on both sides by Ad5 sequence, in a vector containing LEU2 and CYH2 for selection and counter-selection in yeast.

LGM YAC/BAC (pMB26): The E1B::GFP fusion in pMB25 was inserted in the Ad5 YAC/BAC by two step gene replacement. The resulting plasmid was transferred to E. coli to allow production of enough DNA for sequencing and transfection into mammalian cells. Plasmid from E. coli was cut with PacI to liberate the Ad 5 insert, then transfected into 293 cells to make virus.

E2-Tcfx3/4 mutations (pMB33/69): An Ad5 fragment containing the E2 region was cloned into a vector containing URA3 for selection and counterselection in yeast. The E2 promoter was replaced by inverse PCR. pMB33 contains 3 Tcf sites; pMB69 contains 4 Tcf sites.

transferred by two step gene replacement. pMB73 lacks the ATF site mutations because the ATF site is the nearest to the site of integration.

Detailed procedures:

pMB20: Ad5 genomic DNA was gap repaired into pMB19 cut with SalI. pMB19 was made by inserting a yeast replication origin (from pH4ARS, Bouton and Smith, 1986) into the SacI site of a vector already containing the terminal Ad5 fragments (pMB10). The starting vector (pNKBAC39, Larionov et al., 1996) was expected not to need an ARS but this assumption proved incorrect. The Ad5 terminal fragments were cloned initially by PCR into a pUC19-derived vector (to give pMB1 and pMB2), and then transferred sequentially into the BamHI/Bsu36I sites of pNKBAC39. PacI sites were present in the G76 primer used to make pMB1 and pMB2 (PCR with primers G74-G76 and G75-G76 giving Ad5 fragments of 390 and 356 bp).

pMB25: The EGFP vector is a modified vector from Clontech. It has the 5' end of GFP from pEGFP-C1 and the 3'-end of GFP from phGFP-S65T. The Ad5 PCR fragment (nt 2019-2261, primers G77-78) was cloned into the NheI and AgeI sites at the 5'-end of EGFP to give pMB7. The SmaI Ad5 fragment (nt 1007-3940) containing the E1 region was cloned into Bluescript to give pMB22. The E1B::GFP fusion (NotI/KpnI) was cloned into pMB22 (BglII/KpnI) to give pMB24. The XhoI/BamHI fragment of pMB24 containing the Ad5 insert was cloned into p680 (Ketner et al., 1994) to give pMB25.

pMB33: The Ad5 PCR fragment (nt 26688-27593, PCR with primers G61-G62, product cut with SacI/KpnI) was cloned into the KpnI/SacI sites in pRS406 to give pMB32. This was mutagenised by inverse PCR to insert the Tcf sites using primers G63-G64. The primers should give four Tcf sites but the first Tcf site was subsequently found to contain a mutation, so the final vector only contains three Tcf sites.

G74 Ad5, 390 (left arm gap repair fragment)
5'-GGG CGA GTC TCC ACG TAA ACG-3'

G75 Ad5, 36581 (right arm gap repair fragment)
5'-GGG CAC CAG CTC AAT CAG TCA-3'

G76 Ad5 ITR plus EcoRI, HindIII and PacI sites
5'-CGG AAT TCA AGC TTA ATT AAC ATC ATC AAT AAT ATA CC-3'

G77 Ad 5, 2020 (E1B fragment plus NheI site)
5'-GCG GCT AGC CAC CAT GGA GCG AAG AAA CCC A-3'

G78 Ad 5, 2261 (E1B fragment plus AgeI site)
5'-GCC ACC GGT ACA ACA TTC ATT-3'

G87 iPCR to destroy the E3 NF-1, L1 and L2 binding sites, upper primer
5'-AGCTGGGCTCTCTTGGTACACCAGTGCAGCGGGCCAACTA-3'

G88 iPCR to destroy the E3 NF-1, L1 and L2 binding sites, lower primer
5'-CCCACCACTGTAGTGCTGCCAAGAGACGCCCAGGCCGAAGTT-3'

G89 iPCR to destroy the E3 ATF and AP-1 binding sites, upper primer
5'-CTGCGCCCCGCTATTGGTCATCTGAACTTCGGCCTG-3'

G90 iPCR to destroy the E3 ATF and AP-1 binding sites, lower primer
5'-CTTGCGGGCGGCTTTAGACACAGGGTGCGGTC-3'

G91 iPCR, E2 promoter replacement (1 x Tcf), upper primer
5'-CAGATCAAAGGGCCATTATGAGCAAG-3'

G92 iPCR, E2 promoter replacement (1 x Tcf), lower primer

3 Tcf sites:
GACTAG-ATCAAGGGTTGGAGATCAAAGGGATCCAGATCAAAGGGATTAA
GAT CAAAGG-GCCATT

4 Tcf sites:
GACTAG-ATCAAAGGGTTGGAGATCAAAGGGATCCAGATCAAAGGGATTA
AGATCAAAGG-GCCATT

Note: The 3 Tcf vector is a mutant form of the 4 Tcf vector resulting from a PCR cloning artefact (there is a single A deletion in the first Tcf site). This is the sequence present in the Ad-Tcf3 and LGC viruses.

E3 promoter binding sites

Four sites have been identified in the E3 promoter by DNase I foot-printing in Hela cells (Garcia 1987, Hurst 1987) Site 1 covers the TATA box, the remaining sites (underlined and marked H2-H4 in Figures 1 to 3) are bound by ATF, AP1 and NF1. Two sites have been identified by DNase I footprinting in lymphoid cells (Williams 1990) (underlined and marked L1 and L2 in Figures 1 to 3), they bind NFκB family members.

To inactivate the promoter, mutations were introduced by inverse PCR. All sites except the TATA box contain at least one mutation. The mutations are silent at the protein level. The L1, L2 and H2 boxes contain multiple substitutions of highly conserved residues. The H4 box contains a single mutation in a relatively poorly conserved residue because of the limited choice of alternative codons. Mutation of the H4 site was not a priority because deletion of this site has no effect on E3 transcription in Hela cells (Garcia 1987). Only relatively weakly conserved residues in the H3 box were mutated because any modification of the most conserved residues in the published AP1 site (TGAC) would change the protein sequence. There is a better match to an AP1 site slightly 3' of the published site (still within the published footprint); the mutations introduced completely destroy this site.

vMB17 = virus pool derived from pRDI-254 transfection

vMB18 = virus pool derived from pRDI-264 transfection

6. The viruses were plaque purified on SW480 cells because these cells contain active Tcf and have no endogenous E1B sequences with which the viral genome could recombine. The viruses were then expanded on SW480, purified by Cs banding, and checked by restriction digestion and sequencing in the E1B and E2/E3 regions. The plaque purified viruses were given the following names:

vMB23 = Ad5 with 4 x Tcf sites in the E1B promoter

vMB27 = Ad5 with 4 x Tcf sites in both the E1B and E2 promoters

vMB31 = Ad5 with 4 x Tcf sites in the E1B and E2 promoters, and a mutant E3 promoter with a wild type ATF site

vMB19 = Ad5 with 4 x Tcf sites in the E1B and E2 promoters, and a fully mutant E3 promoter

RESULTS:

Luciferase assays using Tcf reporters show that p53-mutant lung carcinoma cells (H1299) lack Tcf activity and p53-mutant colon carcinoma cells (SW480) have strong Tcf activity. Viruses selective for cells containing Tcf activity should therefore replicate in SW480 but not H1299. The inventors have demonstrated that matched viruses with Tcf-mutant E2 promoters express E2 gene products preferentially by western blotting; replicate better by slot blotting and quantitative PCR; and have greater cytopathic effect in SW480 than H1299. The relatively modest effect of an E2 promoter mutation alone is considerably enhanced when the virus also lacks E1B 55k. E1B 55k mutations reduce nuclear export of DBP mRNA transcribed from the E2 late promoter. DBP can be expressed from both the early and late E2 promoters. The inventors have determined that the E1B 55k-dependent reduction in DBP expression might be rescued by the Tcf mutations in the E2 early promoter. Consistent with this the level of DBP expressed from LGC is significantly reduced in H1299. This effect

These results are consistent with the stronger expression of pol and pTP in Tcf-mutant E2 promoter viruses in colon cells resulting in greater viral replication. Additionally, the data show that the combination of an E2 promoter mutation with deletion of E1B 55k results in exceptionally little replication in cells lacking Tcf activity.

Cytopathic effect (CPE) assays

H1299 and SW480 were infected with five-fold dilutions of wild type Ad5, Ad-Tcf3, LGM and LGC (moi of 0.6 in well 1, moi of 0.001 in well 5). Dishes were stained with crystal violet after eight days. CPE is apparent with wild type Ad5 in H1299 even at the highest dilution. Wild type Ad5 is five-fold less active in SW480 than H1299.

Ad-Tcf3 is five-fold less active than wild type Ad5 in H1299. LGM is 125-fold less active than wild type Ad5 in H1299. LGC is 625-fold less active than wild type Ad5 in H1299. The Tcf3 mutation in the E2 promoter thus results in a five-fold reduction in CPE in H1299. Ad-Tcf3 is similar to wild type Ad5 in SW480, indicating that there is a five-fold gain in activity in cells containing Tcf activity. LGC is five-fold more active than LGM in SW480.

LGC is 625-fold less active than wild type Ad5 in cells lacking Tcf activity, but only five-fold less active in cells containing Tcf activity. This represents a 125-fold selectivity for colon cells, and a five-fold greater activity than a simple E1B 55k-deficient virus.

Additional CPE assays were performed with SW480 cells and normal lung fibroblasts using the above viruses Ad-Tcf3, LGM and LGC. Ad-Tcf3 gave wild type activity on SW480 but 5-fold less activity on normal fibroblasts. In this experiment LGM and LGC both gave about 125-fold less activity than wild type Ad5 on SW480 but 5-fold and >125-fold less activity respectively on normal fibroblasts.

The selectivity and efficacy of Ad-Tcf 3 are shown to be greater than that of LGM which itself has essentially the same properties as the ONYX-015 virus.

E3 reverse primer: AACACCTGGTCCACTGTCGC
E3 probe: CCGCGACTCCGTTTCAACCCAGA
E1B-55k forward primer: TGCTTCCATCAAACGAGTTGG
E1B-55k reverse primer: GCGCTGAGTTTGGCTCTAGC
E1B-55k probe: CGGCGGCTGCTCAATCTGTATCTTCA
E4 forward primer: GGTTGATTCATCGGTCAGTGC
E4 reverse primer: ACGCCTGCGGGTATGTATTC
E4 probe: AAAAGCGACCGAAATAGCCCG
Fibre forward primer: TGATGTTTGACGCTACAGCCATA
Fibre reverse primer: GGGATTTGTGTTTGGTGCATTAG

Western Blot analysis

Cells were infected with either 300 (SW480 and H1299) or 1000 (WI38) viral particles/cell in DMEM 10 % FCS. Two hours after infection, the medium was removed and replaced with 2 ml of fresh DMEM 10 % FCS. Cells were harvested 24 hours after infection. Immunoblotting was performed as described by Harlow and Lane (Antibodies: A laboratory manual, New York: Cold Spring Harbor Laboratory Press, 1988 incorporated herein by reference). Dr. A. Levine provided both 2A6 anti-E1B55K antibody (Sarnow P. et al, Virology, 1982, 120: 510-517) and B6 anti-DBP antibody (Reich N. et al, Virology, 1983, 128: 480-484). Detection was with HRP-conjugated rabbit anti-mouse IgG (DAKO A/S, Denmark) and chemiluminescence (Amersham, Little Chalfont, UK)

Plaque assay

Cells were infected in duplicate with either 300 (for SW480 and H1299) or 1000 (WI38) viral particles/cell in DMEM 10 % FCS. After two hours incubation at 37 °C, the medium was removed and replaced with 2 ml of fresh DMEM 10 % FCS. After an additional 48 hours, the cells were scraped into the culture medium and lysed by three cycles of freeze-thawing. The supernatant of each duplicate point was tested

Taqman RT-PCR transcription assays (see Fig 10) show mutant viruses to have wild type levels of E2 mRNA in SW480 cells, but reduced levels in H1299 cells:

The Taqman assay further demonstrates that mutation of the E3 promoter in vMB14 decreases both E2 (Fig 10) and E3 mRNA levels (Fig 11):

To determine whether DNA replication is affected by the promoter mutations, SW480 and H1299 cells were infected with wild type, vMB12, 13 and 14, and harvested at 24 hours. Sybr green PCR assays using primers from the fibre region show that DNA replication is normal in SW480 but reduced in H1299 cells (Fig 12):

To determine whether virus replication is affected by the promoter mutations, SW480 and H1299 cells were infected with wild type, vMB12, 13 and 14, and harvested at 48 hours. Cells were lysed by freeze-thawing and virus production was measured by plaque forming assay on SW480 cells. This showed that the mutant viruses are comparable or better than wild type in SW480 cells, but defective in H1299 and WI38 cells (See Fig 13):

To determine whether the viruses show selective toxicity to colon cells, cytopathic effect assays were performed on SW480 and H1299 cells. This showed that the mutant viruses are comparable to wild type in SW480 cells (Fig 14) but showed reduced cytopathic effect in H1299 cells (Fig 15):

To determine whether the viruses show a therapeutic effect in vivo, they were injected into Co115 colon carcinoma xenografts in nude mice. This showed that intratumoral injection of all of the viruses delay the growth of xenografted colon tumours and prolong the survival of nude mice. vMB12 and 13 were more effective than vMB14 (Fig 16):

E1B expression is required for induction of inflammatory damage by adenoviruses in cotton rat lung (Ginsberg, H. S., et al 1999. PNAS *96*, 10409-11). To reduce the risk of inflammatory reactions, Tcf sites were cloned into the E1B promoter. The resulting viruses should express E1B gene products in colon tumour cells but not in normal cells. In addition to reducing the risk of inflammatory reactions, this could also reduce expression of E2 gene products from the E2 late mRNA, because E1B 55k is reported to be required for E2 late mRNA export. The

5. the mutant viruses impair the growth of xenografts in nude mice

6. insertion of Tcf sites in the E1B promoter is compatible with the production of viable adenovirus

7. the mutant E1B promoter is more active in colon tumour cells than non-colon tumour cells

8. in the models tested so far the E1B promoter mutation does not affect virus replication

9. the virus with combined E1B and E2/E3 promoter mutations shows reduced replication in cotton rat lungs, and is expected to produce less inflammatory damage than viruses with the E2/E3 mutations alone.

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Xie, J., Murone, M., Luoh, S. M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. W., Hynes, M., Goddard, A., Rosenthal, A., Epstein, E. H., Jr., and de Sauvage, F. J. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature 391, 90-2.

4. A construct as claimed in Claim 2 or Claim 3 characterised in that its nucleic acid sequence corresponds to that of the genome of an adenovirus, lentivirus, polyomavirus, vaccinia virus, herpes virus or parvovirus with the selected transcription factor binding sites operatively positioned to control expression of the early viral genes.

5. A construct as claimed in any one of the preceding claims characterised in that its nucleic acid sequence, other than the selected sites, corresponds to that of the genome of adenovirus Ad5, Ad40 or Ad41, or incorporates DNA encoding for fibre protein from Ad 5, Ad40 or Ad41, optionally with 15 to 25 lysines added to the end thereof.

6. A construct as claimed in any one of the preceding claims characterised in that the genes controlled by the tumour specific transcription factor binding site are DNA polymerase, DNA terminal protein and/or DNA binding protein.

7. A construct as claimed in any one of the preceding claims characterised in that its nucleic acid sequence corresponds to that of an adenovirus having a wild type E2 early promoter transcription factor binding site replaced by the tumour cell specific transcription factor binding site.

8. A construct as claimed in any one of the preceding claims characterised in that it encodes a functional viral RNA export capacity.

9. A construct as claimed in any one of the preceding claims having an E1 region wherein the E1B 55K gene is functional and/or intact.

10. A construct as claimed in any one of the preceding claims characterised in that the tumour specific transcription factor binding site used in place of wild type site is selected from Tcf-4, RBPJκ, Gli-1, HIF1alpha and telomerase promoter binding sites.

19. A construct as claimed in any one of the preceding claims characterised in that its sequence corresponds to that of an adenovirus genome wherein the E2 late promoter has been inactivated with silent mutations.

20. A virus comprising or encoded by a DNA construct as claimed in any one of Claims 1 to 19.

21. A virus encoding only wild type proteins other than one or more optional non-wild type non-cytotoxic marker proteins, for use in therapy.

22. A viral DNA construct, or a virus, as claimed in any one of Claims 1 to 20 for use in therapy.

23. A viral DNA construct, or a virus, as claimed in Claims 21 or Claim 22 characterised in that the therapy is of patients having neoplasms.

24. Use of a viral DNA construct, or a virus, encoding only wild type proteins, other than one or more optional non-wild type non-cytotoxic marker proteins, in the manufacture of a medicament for the treatment of neoplasms.

25. Use of a viral construct, or a virus, as claimed in any one of Claims 1 to 22 in the manufacture of a medicament for the treatment of neoplasms.

26. A pharmaceutical composition comprising a viral DNA construct, or a virus encoded thereby, encoding only wild type proteins other than one or more optional non-wild type non-cytotoxic marker proteins, together with a physiologically acceptable carrier, wherein the composition is sterile and pyrogen free with the exception of the presence of the DNA or virus encoded thereby.

35. A method for treating a patient suffering from neoplasms wherein a virus encoding only wild type proteins other than one or more optional non-wild type non toxic-marker proteins is caused to infect tissues of the patient, including or restricted to those of the neoplasm, and allowed to replicate such that neoplasm cells are caused to be killed.

36. A method for treating a patient suffering from neoplasms wherein a viral DNA construct or virus as claimed in any one of Claims 1 to 22 is caused to infect tissues of the patient, including or restricted to those of the neoplasm, and allowed to replicate such that neoplasm cells are caused to be killed.

Fig.1 (Cont).

```
421
ACC CGA ATA AAC TAC ATG AGC GCG GGA CCC CAC ATG ATA TCC CGG GTC AAC GGA ATC CGC
T   R   I   N   Y   M   S   A   G   P   H   M   I   S   R   V   N   G   I   R

481
GCC CAC CGA AAC CGA ATT CTC TTG GAA CAG GCG GCT ATT ACC ACC ACA CCT CGT AAT AAC
A   H   R   N   R   I   L   L   E   Q   A   A   I   T   T   T   P   R   N   N

541                 NF1                                 NFκB
CTT AAT CCC CGT AGT TGG CCC GCT GCC CTG GTG TAC CAG GAA AGT CCC GCT CCC ACC ACT
L   N   P   R   S   W   P   A   A   L   V   Y   Q   E   S   P   A   P   T   T
                        H4                          L2

601   NFκB                                    AP1
GTG GTA CTT CCC AGA GAC GCC CAG GCC GAA GTT CAG ATG ACT AAC TCA GGG GCG CAG CTT
V   V   L   P   R   D   A   Q   A   E   V   Q   M   T   N   S   G   A   Q   L
        L1                                      H3

661               ATF                                 E3 TATA box
GCG GGC GGC TTT CGT CAC AGG GTG CGG TCG CCC GGG CAG GGT ATA ACT CAC CTG ACA ATC
A   G   G   F   R   H   R   V   R   S   P   G   Q   G   I   T   H   L   T   I
              H2

721          ⌈E3 transcription start site→
AGA GGG CGA GGT ATT CAG CTC AAC GAC GAG TCG GTG AGC TCC TCG CTT GGT CTC CGT CCG
R   G   R   G   I   Q   L   N   D   E   S   V   S   S   S   L   G   L   R   P
```

Fig.2 (Cont).

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ACC CGA ATA AAC TAC ATG AGC GCG GGA CCC CAC ATG ATA TCC CGG GTC AAC GGA ATC CGC
T   R   I   N   Y   M   S   A   G   P   H   M   I   S   R   V   N   G   I   R

481
GCC CAC CGA AAC CGA ATT CTC TTG GAA CAG GCG GCT ATT ACC ACC ACA CCT CGT AAT AAC
A   H   R   N   R   I   L   L   E   Q   A   A   I   T   T   T   P   R   N   N

541                    NF1                                  NFκB
CTT AAT CCC CGT AGT TGG CCC GCT GCa CTG GTG TAC CAa GAg AGc CCa GCT CCC ACC ACT
L   N   P   R   S   W   P   A   A   L   V   Y   Q   E   S   P   A   P   T   T
                            H4                      L2

601   NFκB                                    AP1
GTa GTg CTg CCa AGA GAC GCC CAG GCC GAA GTT CAG ATG ACc AAt agc GGG GCG CAG CTT
V   V   L   P   R   D   A   Q   A   E   V   Q   M   T   N   S   G   A   Q   L
      L1                                        H3

661                ATF                          E3 TATA box
GCG GGC GGC TTT aGa CAC AGG GTG CGG TCG CCC GGG CAG GGT ATA ACT CAC CTG ACA ATC
A   G   G   F   R   H   R   V   R   S   P   G   Q   G   I   T   H   L   T   I
              H2

721          ⌈E3 transcription start site→
AGA GGG CGA GGT ATT CAG CTC AAC GAC GAG TCG GTG AGC TCC TCG CTT GGT CTC CGT CCG
R   G   R   G   I   Q   L   N   D   E   S   V   S   S   S   L   G   L   R   P
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Fig.3 (Cont).

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421
ACC CGA ATA AAC TAC ATG AGC GCG GGA CCC CAC ATG ATA TCC CGG GTC AAC GGA ATC CGC
T   R   I   N   Y   M   S   A   G   P   H   M   I   S   R   V   N   G   I   R

481
GCC CAC CGA AAC CGA ATT CTC TTG GAA CAG GCG GCT ATT ACC ACC ACA CCT CGT AAT AAC
A   H   R   N   R   I   L   L   E   Q   A   A   I   T   T   T   P   R   N   N

541                     NF1                            NFκB
CTT AAT CCC CGT AGT TGG CCC GCT GCC CTG GTG TAC CAG GAA AGT CCC GCT CCC ACC ACT
L   N   P   R   S   W   P   A   A   L   V   Y   Q   E   S   P   A   P   T   T
                            H4                          L2

601   NFκB                                               AP1
GTG GTA CTT CCC AGA GAC GCC CAG GCC GAA GTT CAG ATG ACT AAC TCA GGG GCG CAG CTT
V   V   L   P   R   D   A   Q   A   E   V   Q   M   T   N   S   G   A   Q   L
        L1                                          H3

661             ATF                                   E3 TATA box
GCG GGC GGC TTT CGT CAC AGG GTG CGG TCG CCC GGG CAG GGT ATA ACT CAC CTG ACA ATC
A   G   G   F   R   H   R   V   R   S   P   G   Q   G   I   T   H   L   T   I
            H2

721             ⌈E3 transcription start site→
AGA GGG CGA GGT ATT CAG CTC AAC GAC GAG TCG GTG AGC TCC TCG CTT GGT CTC CGT CCG
R   G   R   G   I   Q   L   N   D   E   S   V   S   S   S   L   G   L   R   P
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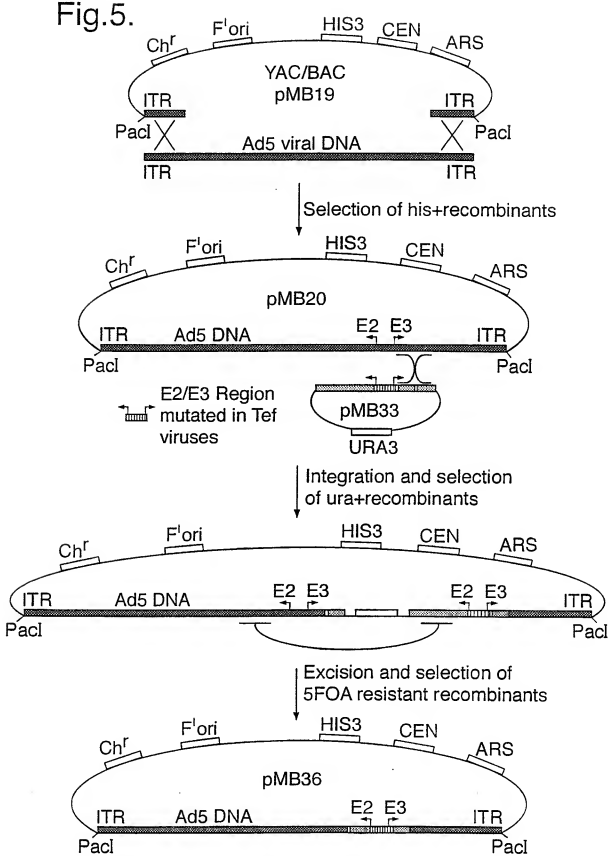
Fig.5.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E2 E3
E2/E3 Region
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pMB36

Fig.7.

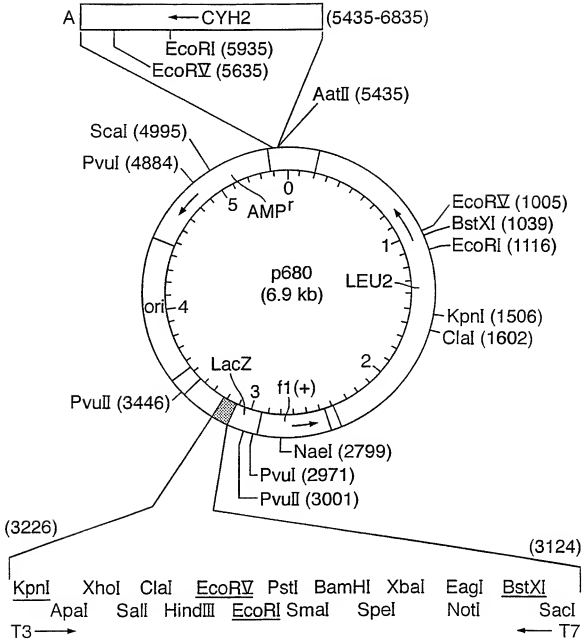
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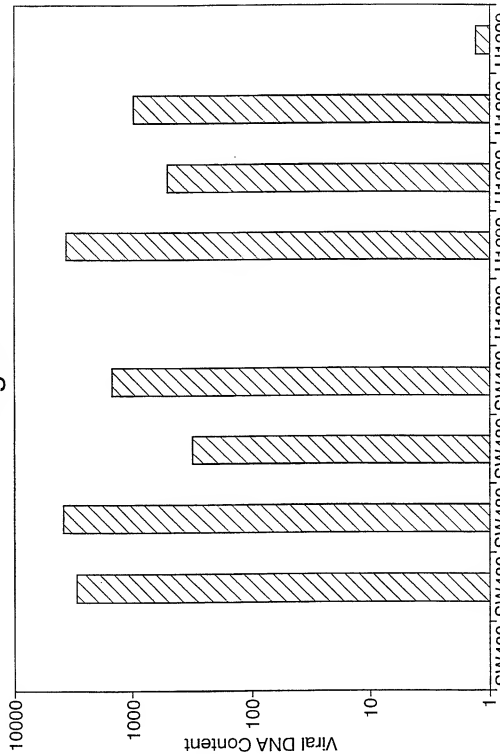
Fig.9.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100
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Viral DNA Content
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SW480 wt
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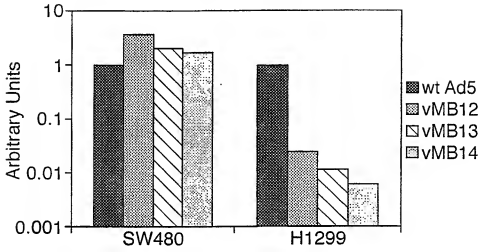
H1299 LGC
Cell Line/Virus

Fig.12.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vMB14

Fig.13.

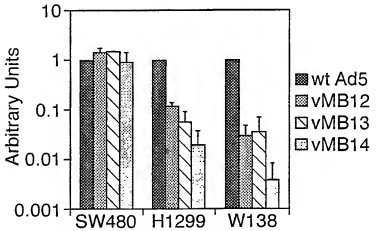
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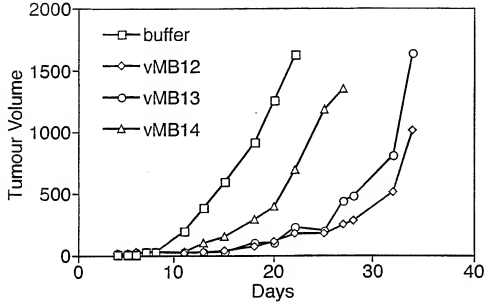
Fig.16.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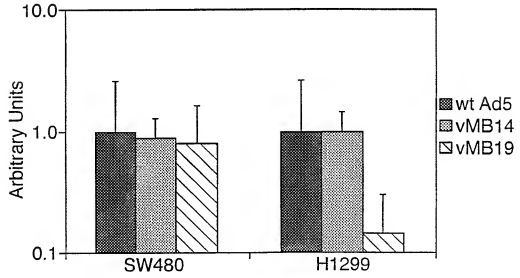
Days

Fig.17.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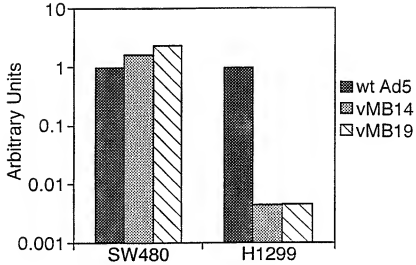
SW480
H1299

Fig.20.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Gly Leu Arg Pro
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<220>
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<400> 45
gggatttgtg tttggtgcat tag 23

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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PCT/GB 00/01142

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